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REGULAR ARTICLE

Competition for nitrogen between *Pinus sylvestris* and ectomycorrhizal fungi generates potential for negative feedback under elevated CO₂

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Abstract We investigated fungal species-specific responses of ectomycorrhizal (ECM) Scots pine (*Pinus sylvestris*) seedlings on growth and nutrient acquisition together with mycelial development under ambient and elevated CO₂. Each seedling was associated with one of the following ECM species: *Hebeloma cylindrosporum*, *Laccaria bicolor*, *Suillus bovinus*, *S. luteus*, *Piloderma croceum*, *Paxillus involutus*, *Boletus badius*, or non-mycorrhizal, under ambient, and elevated CO₂ (350 or 700 µl l⁻¹ CO₂); each treatment contained six replicates. The trial lasted 156 days. During the final 28 days, the seedlings were labeled with ¹⁴CO₂. We measured hyphal length, plant biomass, ¹⁴C allocation, and plant nitrogen and phosphorus concentration. Almost all parameters were significantly affected by fungal species and/or CO₂. There were very few significant interactions. Elevated CO₂ decreased shoot-to-root ratio, most strongly so in species with the largest extraradical mycelium. Under elevated CO₂, ECM root growth increased significantly more than hyphal

growth. Extraradical hyphal length was significantly negatively correlated with shoot biomass, shoot N content, and total plant N uptake. Root dry weight was significantly negatively correlated with root N and P concentration. Fungal sink strength for N strongly affected plant growth through N immobilization. Mycorrhizal fungal-induced progressive nitrogen limitation (PNL) has the potential to generate negative feedback with plant growth under elevated CO₂.

Keywords Carbon and nitrogen allocation · Ectomycorrhizal fungi · Elevated CO₂ · Extraradical hyphae · Mycogenic · N immobilization · Progressive nitrogen limitation · *Pinus sylvestris*

Introduction

Responses to elevated CO₂ have often been studied from the plant's perspective (phytogenic approach) and less often from the fungal perspective (mycogenic approach) in which carbon and nutrient sink strength of the fungus plays an important role (Fitter et al. 2000; Staddon 2005). It has often been assumed that plant and fungal responses are coordinated and of the same magnitude, implying that elevated CO₂ provides roughly similar benefits for both symbiotic partners (Alberton et al. 2005; Reich et al. 2006). Under such conditions positive feedbacks between increased carbon availability and increased nutrient availability are likely to occur, leading to sustained

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increased productivity of the plant. However, Diaz et al. (1993) hypothesized that an enhanced C flow to the soil increases nutrient immobilization by (saprotrophic) microbiota and hence decreases plant nutrient availability, which provides a negative feedback and limits plant response to elevated CO₂. Such microbially induced progressive nitrogen limitation (PNL) potentially decreases plant response to rising atmospheric CO₂ (Luo et al. 2004; Hu et al. 2006). Furthermore, a recent meta-analysis of mycorrhizal plant and fungal responses to elevated CO₂ suggested that in ectomycorrhizal (ECM) symbioses, fungal responses are larger than plant responses (Alberton et al. 2005). On average, doubling atmospheric CO₂ concentration led to an increase of 45% of the ECM mycelium and 26% of the plant. As a consequence of this differential response, competition between mycorrhizal plant and fungus for nutrients could increase.

The meta-analysis also indicated that different fungal species derive similar benefits from elevated CO₂, but that fungal species identity matters very much from the plant's perspective. Plants associated with individual fungal species also showed a significantly lower response to elevated CO₂ than plants that were colonized by a fungal assemblage. Alberton et al. (2005) proposed two hypotheses to explain this observation. Elevated CO₂ could result in a shift in the fungal assemblage toward species that have lower nutrient-to-carbon demands (Fransson et al. 2001; Parrent et al. 2006). The alternative hypothesis is that research results are biased by selection of species that perform well under the conditions normally encountered in experiments, but that are not sufficiently representative for fungal assemblages in the field. Research bias likely leads to overrepresentation of ECM fungi that tend to be nutrient-limited rather than carbon-limited, since ECM fungi contain higher concentration of N than plant material (Colpaert et al. 1996). Because the meta-analysis indicated that fungal species identity was a major determinant of the outcome of individual studies, Alberton et al. (2005) also suggested argued choices in species selection for experiments. Because only a limited number of species have been investigated to date, there is a clear need for testing ECM plant and fungal responses to elevated CO₂ by a number of different fungal species, with different carbon and nutrient economies, in one experiment (see also Fransson et al. 2007).

This work aimed at investigating fungal species-specific responses of ECM Scots pine seedlings under controlled conditions at ambient and elevated CO₂. We hypothesized that (1) elevated CO₂ will increase carbon allocation to the fungus; (2) enhanced allocation will increase nutrient immobilization in fungal tissue; (3) larger nutrient immobilization in fungal tissue will show negative feedback to plant nutrient content and ultimately plant biomass due PNL; (4) under such conditions mycorrhizal associations may constrain plant responses to elevated CO₂; (5) these effects will be strongest in species that produce the largest amount of mycelium. In other words, this study sought for evidence of nutrient competition and hence PNL between ECM fungus and plant in order to improve our understanding and predictive ability of ECM functioning under elevated CO₂.

Materials and methods

Plants, fungi, and substrate

Seeds of Scots pine (*Pinus sylvestris*), purchased from PVM, Grubbenvorst, The Netherlands, were soaked in demineralized water overnight, and then surface-sterilized in a 30% H₂O₂-solution containing a drop of Tween 20 during 30 min. The sterilized seeds were germinated on sterile water agar media containing 5 g l⁻¹ of glucose. After 3 weeks, each seedling was transferred to a Petri dish (Ø 14.5 cm), filled with 50 g of a sterilized (1 h at 110°C) peat-vermiculite mixture (2:5, w/w). The mixture contained 29.9 mg kg⁻¹ of available mineral N and 1.4 mg kg⁻¹ of available P. The peat-vermiculite mixture was moistened with 50 ml full-strength modified Melin–Norkrans (MMN) solution (without agar, glucose, and malt extract) at the start of the experiment. MMN solution was used to ensure that both mycorrhizal and non-mycorrhizal treatments were solely limited by N (and not by P), and to prevent a decrease in pH due the ammonium-N uptake during the experiment (Kamminga-van Wijk and Prins 1989). During the experiment we furthermore added five times 23 ml MMN. In total, 8.66 mg of N and 28.48 mg of P were added to each Petri dish. Each seedling was inoculated around the root system with 12 plugs of actively growing mycelium of one of

the following ECM species: *Hebeloma cylindrosporum* (CBS 558.96, kindly provided by Centraalbureau voor Schimmelcultures CBS, Utrecht, The Netherlands), *Laccaria bicolor*, *Suillus bovinus*, *S. luteus*, *Piloderma croceum* (BL 97-01, kindly provided by R. Finlay, SLU, Uppsala, Sweden), *Paxillus involutus*, *Boletus badius*. A non-mycorrhizal treatment was also included. The isolates of *L. bicolor*, *S. bovinus*, *S. luteus*, *P. involutus*, and *B. badius* were isolated from fruitbodies collected in the surroundings of Harderwijk, The Netherlands, in 2004. The Petri dishes were hermetically sealed with parafilm and tape, covered with aluminum foil, but contained three openings. The median opening allowed shoots to grow out of the dish using lanoline for sealing, while one lateral opening could be instantaneously opened for water addition, after which it was closed again. During the labeling, the third opening was connected to a PVC tube (\varnothing 2 cm; length 15 cm) containing three layers of soda-lime (carbosorb) separated by cotton plugs to trap root and mycorrhizal respiration and to permit the free exchange of oxygen. Approximately 7.5 g of soda-lime were used in the lower layer to capture respiration. The upper layer served as a barrier to capture CO_2 entering from atmosphere, and the layer in between was used as a background.

Plant growth and experimental conditions

The seedlings were placed into four growth chambers (two for each CO_2 level) with a steady flow of either 350 or 700 $\mu\text{l l}^{-1}$ CO_2 . Temperatures were maintained at 20°C during the day (16 h, irradiation 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and at 16°C during the night (8 h), and relative humidity was kept around 70%. The Petri dishes were randomized every 2 weeks within each growth chamber. After 128 days, the Petri dishes were transferred to the Experimental Soil Plant Atmosphere System (ESPAS; Gorissen et al. 1996). The seedlings were exposed to $^{14}\text{CO}_2$ (specific radioactivity $0.35 \pm 0.05 \text{ kBq mg}^{-1} \text{ C}$) supplied from a pressurized cylinder for an additional 28 days. The growth conditions (temperature, relative humidity, and CO_2 level) in the ESPAS were equal to the growth chambers, except for the photosynthetically active irradiation level, which was increased to 360 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the first 2 weeks and 460 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the last 2 weeks.

Analyses

All seedlings were harvested 28 days after ^{14}C -labeling started. Plants were removed from Petri dishes; shoots, roots, and the peat-vermiculite mixture were separated and dried at 70°C for 48 h. These fractions were subsequently ground and analyzed for total ^{14}C content with a modified wet combustion method (Dalal 1979). Plant material (30 mg) and peat-vermiculite (0.5 g) were digested in duplicate in 5 ml of a 10% (w/v) solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in a mixture of concentrated H_2SO_4 and H_3PO_4 (3:2, v/v) at 160°C for 2 h. The $^{14}\text{CO}_2$ evolved was trapped in 10 ml of 0.5 M NaOH. The ^{14}C was determined in 0.5 ml of NaOH by liquid scintillation counting (Tri-Carb 2100TR; Packard Instrument Company, Meriden, CT, USA) with 3 ml of Ultima Gold (Packard).

The soda-lime containing the respired $^{14}\text{CO}_2$ was transferred to bottles (0.5 l) and dissolved in 50 ml excess 6 M HCl, injected through a septum in the lid. The $^{14}\text{CO}_2$ evolved was captured in 10 ml of 5 M NaOH and analyzed by liquid scintillation counting as described.

N and P concentrations in shoots and roots were determined colorimetrically by automated flow analysis after digestion of samples with H_2SO_4 –salicylic acid– H_2O_2 and selenium (Temminghoff and Houba 2004).

The total extraradical hyphal length in the peat-vermiculite mixture was determined with the gridline intersection method and fluorescent staining as outlined by Bloem and Vos (2004). We did not subtract hyphal length as measured in the control treatments from hyphal length as measured in the mycorrhizal treatments.

Statistical analyses

The experiment consisted of two CO_2 concentrations (350 or 700 $\mu\text{l l}^{-1}$ CO_2), eight mycorrhizal treatments (seven ECM species and a non-mycorrhizal control) and six replicates for each treatment. A total of 96 seedlings were included ($2 \times 8 \times 6$). In the growth chamber, all Petri dishes for treatment combinations were completely randomized. In order to comply with ANOVA assumptions for homogeneity (Levene's test), the following variables were \log_{10} transformed before analysis: extraradical hyphal length, root dry weight, shoot-to-root dry weight ratio, ^{14}C in shoots,

^{14}C in shoot-to-root ratio, net ^{14}C uptake per unit shoot mass, N concentration in shoots and roots, P concentration in roots, N content in shoots, P content in roots, and N uptake efficiency. ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test was performed using SPSS Version 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA). Considering the large number of parameters measured and calculated (26), a sequential Bonferroni correction was applied to keep the P -value constant at $P < 0.05$. Pearson pairwise correlation coefficients were calculated and their significance determined in two-tailed t -test as indicators of direction of relationship between plant and fungal parameters.

Results

No differences were observed between the results from both growth chambers for each CO_2 level, and they were therefore combined. The respiration measurements showed very low and variable values (^{14}C trapped in respiration usually between 0.5 and 1% of ^{14}C in roots). We concluded that the carbosorb traps were most likely leaky, because respiration data are normally an order of magnitude larger (Gorissen et al. 1991; Andersen and Rygielwicz 1995). Therefore, no data on below-ground respiration and below-ground carbon use efficiency (Gorissen and Kuyper 2000) are reported.

Almost all parameters were significantly affected by CO_2 and/or fungal species. There were very few significant interactions (Table 1). The non-mycorrhizal plants performed equally well or significantly better than some mycorrhizal treatments. Omission of the non-mycorrhizal control did in general not affect the significance of the various parameters (data not shown). The parameters will be discussed in the following order: hyphal length, root, and shoot.

Hyphal length was significantly affected by fungal species, but not by elevated CO_2 . Hyphal length increased by 12% under elevated CO_2 , but the increment was not significant (Table 1). Average hyphal length (for both CO_2 levels) ranged from 54 (*S. bovinus*) to 634 (*H. cylindrosporum*) m g^{-1} substrate (Table 2). Hyphal length in the non-mycorrhizal treatment was on average 29 m g^{-1} substrate. Incorporation of ^{14}C in the substrate (where are roots and external mycelia) followed a fairly

similar pattern but showed more variability (higher coefficient of variation). It was lowest in the non-mycorrhizal control and in the treatment with *S. bovinus*, and highest in the treatments with *H. cylindrosporum*, *L. bicolor*, and *P. croceum* (elevated CO_2 only) (Table 3).

Root dry weight was significantly affected by CO_2 level and fungal species, and not by the interaction (Table 1). Under elevated CO_2 root dry weight increased on average by 52%. Root biomass was highest in the treatment with *P. croceum* and lowest in the treatments with *H. cylindrosporum* and *L. bicolor* (Fig. 1). ^{14}C incorporation in roots was also significantly affected by CO_2 level and fungal species, and not by the interaction. Elevated CO_2 increased ^{14}C activity in roots by 87%. Plants inoculated with *H. cylindrosporum* and *L. bicolor* presented the lowest ^{14}C activity (Table 3). Root dry weight and ^{14}C incorporation in roots were significantly positively correlated ($r = 0.73$, $P < 0.01$). Root N and P concentration were also significantly affected by CO_2 level and fungal species, and not by the interaction. Elevated CO_2 decreased root N concentration on average by 14% and root P concentration on average by 7%. Root N concentrations varied between 6 and 9 mg g^{-1} , and were lowest in the treatment with *P. croceum* and highest in the treatment with *H. cylindrosporum* (Table 4). Because elevated CO_2 increased root dry weight (52%), the total amount of N and P in roots increased under elevated CO_2 by 32 and 41%, respectively. Again, both CO_2 level and fungal species, but not the interaction, were significant. Root N content was highest in the treatment with *P. croceum* and lowest in the treatments with *H. cylindrosporum* and *L. bicolor* (Table 4). Root dry weight was significantly negatively correlated with shoot: root ratio ($r = -0.81$; $P < 0.001$) and root N concentration ($r = -0.91$; $P < 0.001$) (Fig. 2). N uptake efficiency ($\text{mg N content g}^{-1}$ non-mycorrhizal root dry weight or ectomycorrhiza) was significantly affected by CO_2 level and fungal species (Table 1). It ranged between 12 and 16 for ambient CO_2 , and 8 and 11 for elevated CO_2 (Table 4). N uptake efficiency was lower for *P. croceum* than for the other mycorrhizal fungi and non-mycorrhizal treatment.

Shoot dry weight was significantly affected by fungus but not by elevated CO_2 (Table 1). On average shoot dry weight increased under elevated

Table 1 *P*-values from a two-way ANOVA for fungal and plant responses to CO₂ treatments with different fungal species inoculated

Parameters	CO ₂	Fungus	CO ₂ × fungus
Hyphal length (m g ⁻¹ substrate)	0.106	0.000**	0.017
Root dry weight (g)	0.000**	0.000**	0.017
Shoot dry weight (g)	0.186	0.000**	0.099
Total dry weight (g)	0.000**	0.000**	0.009
Shoot-to-root dry weight ratio	0.000**	0.000**	0.300
¹⁴ C in roots (kBq)	0.000**	0.001**	0.314
¹⁴ C in substrate (kBq)	0.029	0.000**	0.002*
¹⁴ C in shoots (kBq)	0.940	0.000**	0.029
¹⁴ C uptake by shoots (mg)	0.000**	0.000**	0.029
¹⁴ C in shoot-to-root ratio	0.000**	0.066	0.089
Net ¹⁴ C uptake (kBq)	0.000**	0.000**	0.042
Net ¹⁴ C uptake per unit shoot mass (kBq mg ⁻¹)	0.000**	0.006*	0.175
N concentration in roots (mg g ⁻¹)	0.000**	0.000**	0.461
N content in roots (mg)	0.000**	0.000**	0.019
N concentration in shoots (mg g ⁻¹)	0.000**	0.000**	0.018
N content in shoots (mg)	0.000**	0.000**	0.001*
Total N uptake (mg)	0.047	0.000**	0.002*
N uptake efficiency (mg N g ⁻¹ root)	0.000**	0.000**	0.188
P concentration in roots (mg g ⁻¹)	0.004*	0.000**	0.202
P content in roots (mg)	0.000**	0.000**	0.115
P concentration in shoots (mg g ⁻¹)	0.001**	0.000**	0.545
P content in shoots (mg)	0.011	0.065	0.018
Total P uptake (mg)	0.000**	0.000**	0.045
N-to-P concentration ratio in roots	0.002*	0.089	0.164
N-to-P concentration ratio in shoots	0.000**	0.000**	0.107
Total N-to-P uptake ratio	0.000**	0.161	0.131

P* ≤ 0.01; *P* ≤ 0.001; Differences in bold are still significant after the sequential Bonferroni correlation to avoid type-I errors

CO₂ by 6%. Shoot dry weight was lowest in the treatment with *H. cylindrosporum* (Table 2). ¹⁴C incorporation in shoots followed the same pattern (it increased under elevated CO₂ by only 3%), and both parameters were significantly correlated (*r* = 0.83; *P* < 0.001). The activity of ¹⁴C in shoots in *H. cylindrosporum*-colonized plants was lowest, whereas the activity of ¹⁴C in shoots in *S. bovinus*-colonized plants was highest (Table 3). Shoot dry weight was significantly negatively correlated with hyphal length (*r* = -0.70; *P* < 0.01; log scale to improve the homogeneity of variances; Fig. 3a).

Shoot N concentration ranged between 8 and 12 mg g⁻¹ under ambient CO₂ and 6–7 mg g⁻¹ under

elevated CO₂ (Table 4). Under elevated CO₂ shoot N concentrations decreased on average by 29%. Shoot N concentrations were highest in plants inoculated with *P. croceum*, and lowest in non-mycorrhizal plants and plants inoculated with *H. cylindrosporum* and *L. bicolor*. P concentration in the shoots was highest in plants colonized by *H. cylindrosporum* and lowest in non-mycorrhizal plants and plants colonized by *L. bicolor* and *P. involutus*. Because shoot dry weight increased under elevated CO₂ and N concentration decreased, shoot N content decreased by 24%. Shoot N was lowest in plants inoculated with *H. cylindrosporum*. Shoot N content was significantly negatively correlated with hyphal length (*r* = -0.57; *P* < 0.02) (Fig. 3b).

Table 2 Extraradical hyphal length (m g⁻¹ substrate) and seedling biomass (g) responses to fungal species and CO₂ treatments

Fungal species	CO ₂ (μl l ⁻¹)	Hyphal length	Root dry weight	Shoot dry weight	Total dry weight	Shoot-to-root ratio
<i>H. cylindrosporum</i>	350	600 ± 270 a	0.37 ± 0.08 g	0.23 ± 0.07 c	0.60 ± 0.15 f	0.61 ± 0.1 abc
	700	669 ± 468 a	0.67 ± 0.14 bcdef	0.26 ± 0.06 bc	0.93 ± 0.19 cdef	0.38 ± 0.05 cd
<i>L. bicolor</i>	350	224 ± 54 ab	0.43 ± 0.05 fg	0.41 ± 0.05 a	0.84 ± 0.05 def	0.97 ± 0.19 a
	700	248 ± 63 ab	0.70 ± 0.09 abcde	0.34 ± 0.06 abc	1.04 ± 0.13 bcde	0.49 ± 0.06 bcd
<i>S. bovinus</i>	350	73 ± 31 cdef	0.47 ± 0.16 efg	0.30 ± 0.08 abc	0.78 ± 0.23 ef	0.68 ± 0.2 ab
	700	35 ± 9 fg	1.03 ± 0.07 ab	0.44 ± 0.03 a	1.46 ± 0.07 a	0.43 ± 0.05 bcd
<i>S. luteus</i>	350	116 ± 51 bcde	0.47 ± 0.06 defg	0.32 ± 0.04 abc	0.78 ± 0.07 ef	0.68 ± 0.12 ab
	700	111 ± 14 bcd	0.72 ± 0.21 abcd	0.35 ± 0.07 abc	1.08 ± 0.23 bcde	0.53 ± 0.22 bcd
<i>P. croceum</i>	350	143 ± 59 bcd	0.85 ± 0.14 abc	0.33 ± 0.04 abc	1.18 ± 0.1 abcd	0.41 ± 0.14 cd
	700	180 ± 64 bc	1.09 ± 0.2 a	0.35 ± 0.07 abc	1.45 ± 0.24 a	0.33 ± 0.07 d
<i>P. involutus</i>	350	54 ± 24 def	0.64 ± 0.17 cdef	0.41 ± 0.08 a	1.05 ± 0.2 bcde	0.69 ± 0.22 ab
	700	70 ± 23 cdef	0.76 ± 0.15 abc	0.39 ± 0.1 ab	1.15 ± 0.22 abcd	0.53 ± 0.11 bcd
<i>B. badius</i>	350	88 ± 57 cdef	0.65 ± 0.23 cdef	0.38 ± 0.06 abc	1.03 ± 0.21 bcde	0.65 ± 0.28 abc
	700	119 ± 55 bcde	0.84 ± 0.09 abc	0.39 ± 0.09 ab	1.22 ± 0.16 abc	0.47 ± 0.1 bcd
Non-mycorrhizal	350	17 ± 8 g	0.59 ± 0.05 cdef	0.39 ± 0.05 ab	0.98 ± 0.08 bcde	0.65 ± 0.08 ab
	700	40 ± 18 ef	0.92 ± 0.13 abc	0.41 ± 0.15 ab	1.33 ± 0.26 ab	0.44 ± 0.1 bcd

Mean values ± SD are shown. Means within a column followed by different letters are significantly different

Total plant dry biomass increased by 33% under elevated CO₂ and once again presented significant differences due to fungal species. Total plant biomass was lowest in the treatment with *H. cylindrosporum* and highest in the treatment with *P. croceum* (Table 2). Elevated CO₂ resulted in an average increase of ¹⁴C incorporation by 46%, with significant differences between species (Table 1). On average, total N uptake increased by 8% under elevated CO₂. It was affected by fungal species and by the interaction fungal species × CO₂, but not by CO₂ (Table 1). Plants colonized by *H. cylindrosporum*, *L. bicolor*, and *S. luteus* showed the lowest total N uptake and plants colonized by *P. croceum* and *B. badius* the highest (Table 4). Total N uptake was also significantly negatively correlated with hyphal length ($r = -0.54$; $P < 0.05$) (Fig. 3c). Under elevated CO₂ P uptake increased by 32%. Total P uptake was also affected by fungal species, but not by the interaction. Total P uptake was lower in plants colonized by *H. cylindrosporum*, *L. bicolor*, and *S. luteus* than in plants colonized by *P. croceum* (Table 5).

Shoot: root ratio was significantly affected by elevated CO₂ and fungal species and not by their interaction. On average, elevated CO₂ reduced shoot: root ratio from 0.67 to 0.45, a decrease of 33%. Shoot: root ratio was lowest in plants inoculated with

P. croceum. Shoot: root ratio and the ratio of ¹⁴C incorporation in shoots and roots were significantly correlated ($r = 0.75$; $P < 0.001$). The ¹⁴C in shoot: root ratio decreased by 52% under elevated CO₂. This decrease, caused by an increased C allocation to the roots compared to the shoots during the final 4 weeks of the experiment, indicated increased sink strength below-ground.

Discussion

In our experiment hyphal length of seven different ECM fungal species did not significantly increase under elevated CO₂. The average increase of 12% was much lower than the value of 34% of the general ECM fungal response (i.e., mycelial dry weight, mycelial area, hyphal length, etc.) reported by Alberton et al. (2005). Our experiment did therefore not provide complete support for our first hypothesis. Plant performance (with regard to N) suggests an explanation for the lack of a fungal response.

Mycorrhizal symbiosis often enhances plant growth due to increased nutrient uptake compared to non-mycorrhizal plants. However, in our experiment the non-mycorrhizal plants performed equally well or significantly better than in some mycorrhizal

Table 3 ^{14}C (kBq) in plant and substrate responses to fungal species and CO_2 treatments

Fungal	CO_2 ($\mu\text{l l}^{-1}$)	^{14}C in shoots (kBq)	^{14}C uptake by shoots (mg)	^{14}C in roots (kBq)	^{14}C in substrate (kBq)	^{14}C in shoot-to-root ratio	Net ^{14}C uptake (kBq)	Net ^{14}C uptake per unit shoot mass (kBq mg^{-1})
<i>H. cylindrosporum</i>	350	12.6 \pm 6.3 bc	41.4 \pm 20.7 abc	15.5 \pm 5.6 e	1.6 \pm 1 bc	0.8 \pm 0.3 abcde	29.7 \pm 11.2 e	0.13 \pm 0.01 abcdef
	700	10.5 \pm 3.1 c	26.8 \pm 8 c	30.8 \pm 8.4 abc	1.5 \pm 1.5 bc	0.3 \pm 0.1 f	42.9 \pm 10.5 cde	0.17 \pm 0.02 ab
<i>L. bicolor</i>	350	22.8 \pm 6.2 ab	75.0 \pm 20.5 a	17.5 \pm 4 de	2.0 \pm 0.4 abc	1.4 \pm 0.5 a	42.3 \pm 5.8 cde	0.10 \pm 0.01 ef
	700	12.0 \pm 3.1 bc	30.5 \pm 7.8 bc	29.8 \pm 5.7 abcd	2.3 \pm 1.7 ab	0.4 \pm 0.1 ef	44.0 \pm 6.2 bcde	0.13 \pm 0.02 abcde
<i>S. bovinus</i>	350	18.1 \pm 4.8 abc	59.4 \pm 15.8 ab	16.8 \pm 3.4 e	0.2 \pm 0.2 bc	1.1 \pm 0.4 abc	35.1 \pm 4.9 e	0.12 \pm 0.02 bcdef
	700	25.4 \pm 7.6 a	64.7 \pm 19.3 a	34.2 \pm 5.6 ab	0.1 \pm 0.1 c	0.8 \pm 0.2 abcde	59.7 \pm 10.8 abc	0.14 \pm 0.02 abcde
<i>S. luteus</i>	350	17.6 \pm 3.6 abc	57.8 \pm 11.9 ab	20.1 \pm 5.1 cde	1.1 \pm 1.6 bc	0.9 \pm 0.3 abcde	38.9 \pm 4.2 de	0.13 \pm 0.02 abcdef
	700	22.9 \pm 10.3 ab	58.3 \pm 26.3 ab	39.2 \pm 6.9 a	0.2 \pm 0.1 c	0.6 \pm 0.3 bcdef	62.3 \pm 9.6 ab	0.18 \pm 0.02 a
<i>P. croceum</i>	350	15.6 \pm 3.7 abc	51.1 \pm 12.2 abc	21.0 \pm 6.4 cde	0.8 \pm 0.3 bc	0.8 \pm 0.3 abcde	37.5 \pm 7 de	0.11 \pm 0.02 cdef
	700	20.1 \pm 7.8 abc	51.2 \pm 19.9 abc	30.4 \pm 3.4 abcd	3.8 \pm 0.8 a	0.7 \pm 0.3 abcde	54.3 \pm 9.5 abcd	0.16 \pm 0.01 abcd
<i>P. involutus</i>	350	19.2 \pm 6.2 abc	62.9 \pm 20.4 a	15.8 \pm 4.5 e	0.7 \pm 1 bc	1.3 \pm 0.5 ab	35.6 \pm 8.9 e	0.09 \pm 0.02 f
	700	21.9 \pm 11.8 ab	55.9 \pm 30.1 abc	36.1 \pm 5.8 ab	2.2 \pm 2.3 abc	0.6 \pm 0.3 bcdef	60.2 \pm 13.3 abc	0.16 \pm 0.03 abcd
<i>B. badius</i>	350	21.4 \pm 5.3 ab	70.4 \pm 17.6 a	23.9 \pm 6.7 bcde	0.5 \pm 0.5 bc	1.0 \pm 0.5 abcd	45.9 \pm 2.1 abcde	0.13 \pm 0.02 abcdef
	700	21.0 \pm 3.5 ab	53.5 \pm 9 abc	41.4 \pm 10.3 a	0.5 \pm 0.7 bc	0.5 \pm 0.2 cdef	62.9 \pm 10.4 a	0.17 \pm 0.05 abc
Non-mycorrhizal	350	21.2 \pm 5.3 ab	69.6 \pm 17.3 a	21.1 \pm 8.2 cde	0.1 \pm 0.1 c	1.1 \pm 0.5 abc	42.5 \pm 10.5 cde	0.11 \pm 0.02 def
	700	19.4 \pm 7.1 abc	49.3 \pm 18.1 abc	42.2 \pm 6.3 a	0.3 \pm 0.1 bc	0.5 \pm 0.2 def	61.8 \pm 9.5 ab	0.16 \pm 0.04 abcd

Mean values \pm SD are shown. Means within a column followed by different letters are significantly different

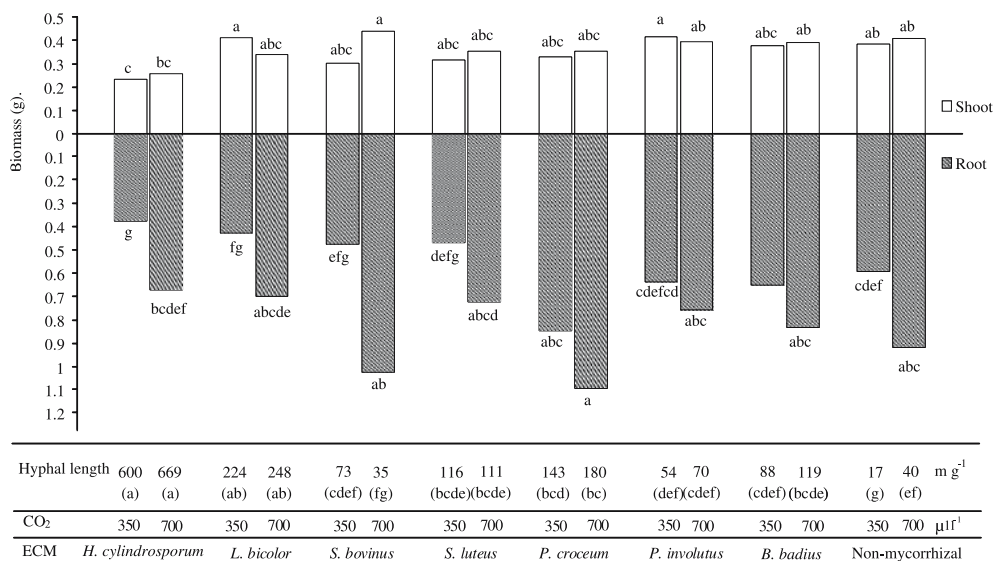


Fig. 1 Main effect of 7 ECM species and non-mycorrhizal treatment under 350 or 700 $\mu\text{l l}^{-1}$ CO₂ on plant shoot and root biomass (g), and extraradical hyphal length (m g^{-1} substrate)

treatments. Especially plants colonized by *H. cylindrosporum* showed a significantly lower growth performance. Basically, two related hypotheses have been forwarded to explain lower growth performance of mycorrhizal plants compared to non-mycorrhizal plants. The first hypothesis focuses on carbon costs for the mycorrhizal fungus, while the second hypothesis focuses on nutrient immobilization by the mycorrhizal fungus.

Under the first hypothesis increased carbon availability (through higher rates of photosynthesis) would decrease relative carbon costs to maintain the mycorrhizal fungus. One corollary following from that hypothesis would be that increased CO₂ availability reduces carbon costs and hence decreases growth reduction. In our experiment, the growth performance of mycorrhizal plants was independent of CO₂ levels. Dry weight of mycorrhizal plants (averaged over seven species) was 91% of that of non-mycorrhizal plants at ambient CO₂ levels and 90% at elevated CO₂ levels. So, increased sink strength for carbon in mycorrhizal plants does not seem to be the cause of the growth reduction. In addition, dry weight of mycorrhizal plants was largely determined by intrinsic fungal properties, notably the length of the extraradical mycelium, because we found significantly negative correlations

values. Letters refer to plant shoot (white bars) and root biomass (shaded bars) and between parenthesis for hyphal length

between extraradical hyphal length and shoot weight (Fig. 3a), shoot N content (Fig. 3b), and total N uptake (Fig. 3c), indicating the importance of fungal sink strength for N.

Several lines of evidence suggest that this growth reduction of mycorrhizal plants was due to N immobilization in the extraradical mycelium. An N balance showed that under ambient CO₂ 76% of the mineral N that was in or added to the system could be accounted for in shoots and roots of non-mycorrhizal plants, and that mycorrhizal colonization reduced that to on average 67%. Under elevated CO₂, 89% of N in the system could be accounted for in shoots and roots of non-mycorrhizal plants, and in ECM plants this was on average 68%. Plants colonized by *H. cylindrosporum*, where a very high length of extraradical mycelium was recorded, remained the smallest, despite the high below-ground investment. Because of the C compounds associated with the immobilization of N, the extraradical mycelium of *Hebeloma* species has been noted to be a large modifier of plant photosynthesis and carbohydrate metabolism (Miller et al. 1989; Rygielwicz and Andersen 1994; Marmesse et al. 1999). Also in conditions of low P supply, *H. cylindrosporum* associated with seedlings of *Pinus pinaster* reduced plant growth due to P retention (Conjeaud et al. 1996). When different kinds of N

Table 4 Shoot and root N concentration (mg g^{-1}), N content (mg), and N uptake efficiency (mg N g^{-1} root) responses to fungal species and CO_2 treatments

Treat	CO_2 ($\mu\text{l l}^{-1}$)	Shoot N concentration	Root N concentration	Shoot N content	Root N content	Total N uptake	N uptake efficiency
<i>H. cylindrosporum</i>	350	8.9 ± 0.3 bcde	9.0 ± 0.9 a	2.0 ± 0.6 cde	3.3 ± 0.7 d	5.4 ± 1.3 e	14.3 ± 1.2 ab
	700	6.0 ± 0.4 fg	7.3 ± 0.8 bcd	1.5 ± 0.4 e	4.9 ± 0.8 abcd	6.4 ± 1.1 de	9.6 ± 0.9 de
<i>L. bicolor</i>	350	8.2 ± 0.9 cde	8.5 ± 0.6 ab	3.3 ± 0.3 ab	3.7 ± 0.3 bcd	7.0 ± 0.5 bcde	16.3 ± 1.8 a
	700	5.6 ± 0.5 g	7.1 ± 0.4 cde	1.9 ± 0.2 de	5.0 ± 0.8 abcd	6.8 ± 0.8 bcde	9.8 ± 0.3 de
<i>S. bovinus</i>	350	9.2 ± 1.6 bcd	7.9 ± 0.9 abc	2.8 ± 0.9 abcd	3.7 ± 1.1 cd	6.5 ± 2 cde	14.0 ± 2.3 ab
	700	7.3 ± 0.9 def	6.6 ± 0.3 de	3.2 ± 0.4 abc	6.8 ± 0.7 a	10.0 ± 1 a	9.7 ± 0.5 de
<i>S. luteus</i>	350	10.1 ± 0.9 abc	8.4 ± 0.9 ab	3.2 ± 0.3 abc	3.9 ± 0.4 bcd	7.1 ± 0.2 bcde	15.2 ± 1.5 a
	700	7.1 ± 0.5 ef	7.3 ± 0.8 bcd	2.5 ± 0.4 bcde	5.2 ± 1 abc	7.7 ± 1 abcde	11.0 ± 2 cd
<i>P. croceum</i>	350	11.6 ± 0.9 a	7.3 ± 0.5 bcd	3.8 ± 0.2 a	6.2 ± 1 a	10.0 ± 1 a	11.9 ± 1.3 bcd
	700	7.1 ± 1.1 ef	6.1 ± 0.5 e	2.5 ± 0.6 bcde	6.7 ± 1.2 a	9.2 ± 1.8 abc	8.5 ± 1 e
<i>P. involutus</i>	350	9.6 ± 1.7 abc	8.2 ± 0.7 abc	3.9 ± 0.6 a	5.2 ± 1.5 abc	9.2 ± 2.1 abcd	14.6 ± 1.7 ab
	700	6.5 ± 0.5 fg	7.3 ± 0.5 bcd	2.5 ± 0.5 bcde	5.5 ± 0.8 ab	8.0 ± 1.2 abcde	10.7 ± 0.9 cd
<i>B. badii</i>	350	10.6 ± 1.5 ab	8.0 ± 0.4 abc	3.9 ± 0.6 a	5.2 ± 1.8 abc	9.1 ± 2.1 abcd	14.6 ± 2.3 ab
	700	7.3 ± 0.8 def	7.5 ± 0.5 bcd	2.8 ± 0.4 abcd	6.3 ± 0.9 a	9.1 ± 1.1 abcd	10.9 ± 0.5 cd
Non-mycorrhizal	350	8.3 ± 0.8 cde	7.8 ± 0.3 abcd	3.2 ± 0.3 abc	4.6 ± 0.4 abcd	7.8 ± 0.6 abcde	13.2 ± 1.2 abc
	700	7.1 ± 0.6 ef	7.0 ± 0.2 cde	2.9 ± 1 abcd	6.3 ± 0.9 a	9.3 ± 1.9 ab	10.0 ± 0.6 de

Mean values \pm SD are shown. Means within a column followed by different letters are significantly different

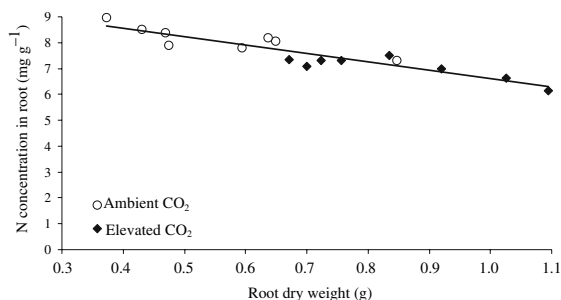


Fig. 2 Relation between root dry weight (g) and root N concentration (mg g^{-1}) under ambient (open circle) and elevated (filled diamond) CO_2 [$n = 16$, $r = -0.908$, and $P < 0.001$]

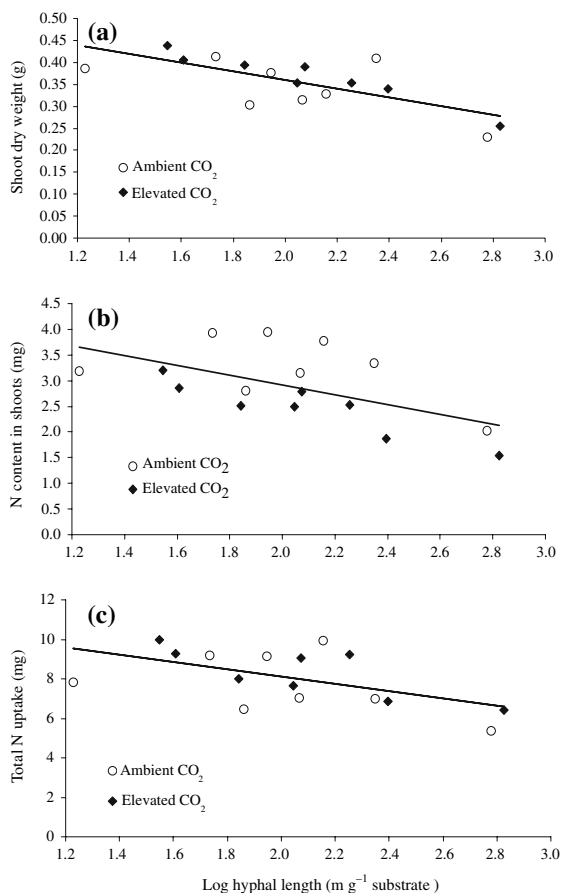


Fig. 3 Relation between hyphal length ($\log \text{m g}^{-1} \text{ substrate}$) and **a** shoot dry weight (g), **b** N content in shoots (mg), and **c** total N uptake (mg) under ambient (open circle) and elevated (filled diamond) CO_2 [$n = 16$, $r = -0.7$, and $P < 0.01$ (a); $r = -0.572$ and $P < 0.02$ (b); $r = -0.54$ and $P < 0.05$ (c)]

were supplied to the symbiosis *P. pinaster*–*H. cylindrosporum*, decreased plant growth of the pine was related to the C cost for fungal growth and N

assimilation, and the extraradical hyphae were considered as the main sink (Plassard et al. 2000). Colpaert et al. (1992) demonstrated a negative correlation between mycelial size and the growth of *P. sylvestris* seedlings and in a subsequent study Colpaert et al. (1996) suggested that growth reduction of pine seedlings colonized by *Sclerotinia citrinum* was best explained by the N-drain.

While in a strict sense our second and third hypothesis were not supported (because under elevated CO_2 there were no increases in the size of the extraradical mycelium), the underlying mechanism of mycorrhizal fungal-induced PNL (mycelial size determines fungal N immobilization potential and hence plant N concentration and content) was confirmed. While laboratory evidence for nutrient (N) retention by ECM fungi resulting in reduced plant growth was also provided by Nylund and Wallander (1989), Dosskey et al. (1990, 1991), Colpaert and Verstuyft (1999), and Fransson et al. (2005), the question is relevant whether the same mechanism could also occur under field conditions. Instances of very strong N-limitation of ECM mycelial growth has been reported from several boreal and arctic ecosystems (Hendricks et al. 2006; Clemmensen et al. 2006; Wallander 2006). There is also field evidence for N-limitation of arbuscular mycorrhizal fungi (Treseder and Allen 2002; Allen et al. 2005). It is therefore likely that mycorrhizal fungal-induced PNL can also occur under field conditions and will ultimately constrain mycorrhizal plant responses to elevated CO_2 (Johnson 2006; Hu et al. 2006).

Further indirect evidence for mycorrhizal-fungal induced PNL comes from N concentration. In our system shoot and root N under ambient CO_2 ranged between 7 and 12 mg g^{-1} , and under elevated CO_2 between 6 and 7 mg g^{-1} . Especially these latter values are clearly below the deficiency limit of 10 mg g^{-1} as mentioned by Reuter et al. (1997) for pines. Similar low plant N concentrations were also observed in northern Finland in Scots pine and Norway spruce (*Picea abies*) stands on very poor soils (Helmisaari et al. 2007) with root N concentrations between 5 and 8 mg g^{-1} , indicating that this degree of N-limitation also occurs in natural systems. The high-N retention by mycorrhizal hyphae apparently limits plant growth given the fact that the non-mycorrhizal plants grew somewhat better than the mycorrhizal plants. This confirms our fourth

Table 5 Shoot and root P concentration (mg g^{-1}), P content (mg), and total P uptake (mg) parameters in responses to fungal species and CO_2 treatments

Treat	CO_2 ($\mu\text{l l}^{-1}$)	Shoot P concentration	Root P concentration	Shoot P content	Root P content	Total P uptake
<i>H. cylindrosporum</i>	350	1.8 ± 0.4 a	4.7 ± 0.5 a	0.40 ± 0.08	1.7 ± 0.3 e	2.2 ± 0.3 e
	700	2.0 ± 0.9 a	3.9 ± 0.3 abc	0.50 ± 0.24	2.6 ± 0.5 bcde	3.1 ± 0.7 bcde
<i>L. bicolor</i>	350	1.4 ± 0.1 ab	4.8 ± 0.6 a	0.58 ± 0.06	2.0 ± 0.3 de	2.6 ± 0.3 de
	700	1.2 ± 0.2 b	4.5 ± 0.3 ab	0.41 ± 0.06	3.1 ± 0.5 abc	3.5 ± 0.5 abcd
<i>S. bovinus</i>	350	2.1 ± 0.9 a	4.4 ± 0.3 abc	0.53 ± 0.16	2.1 ± 0.7 cde	2.6 ± 0.8 de
	700	1.4 ± 0.2 ab	3.7 ± 0.3 abc	0.60 ± 0.1	3.8 ± 0.4 a	4.4 ± 0.4 a
<i>S. luteus</i>	350	1.7 ± 0.4 ab	4.8 ± 0.7 a	0.53 ± 0.06	2.2 ± 0.1 cde	2.7 ± 0.1 de
	700	1.5 ± 0.2 ab	4.2 ± 0.6 abc	0.51 ± 0.09	3.0 ± 0.8 abcd	3.5 ± 0.8 abcd
<i>P. croceum</i>	350	1.7 ± 0.2 ab	3.5 ± 0.4 c	0.54 ± 0.04	2.9 ± 0.2 abcd	3.5 ± 0.2 abcd
	700	1.4 ± 0.3 ab	3.6 ± 0.5 bc	0.50 ± 0.09	3.9 ± 0.5 a	4.4 ± 0.5 a
<i>P. involutus</i>	350	1.7 ± 0.2 ab	4.1 ± 0.7 abc	0.72 ± 0.19	2.5 ± 0.5 bcde	3.3 ± 0.6 abcd
	700	1.2 ± 0.1 b	4.1 ± 0.4 abc	0.48 ± 0.11	3.1 ± 0.5 abcd	3.6 ± 0.5 abcd
<i>B. badius</i>	350	1.9 ± 0.2 ab	4.5 ± 0.6 ab	0.71 ± 0.08	2.8 ± 0.8 abcd	3.5 ± 0.8 abcd
	700	1.3 ± 0.1 b	4.1 ± 0.6 abc	0.51 ± 0.13	3.4 ± 0.7 ab	3.9 ± 0.8 abc
Non-mycorrhizal	350	1.3 ± 0.1 b	3.9 ± 0.3 abc	0.49 ± 0.07	2.3 ± 0.3 cde	2.8 ± 0.3 cde
	700	1.3 ± 0.2 b	3.9 ± 0.3 abc	0.53 ± 0.15	3.6 ± 0.5 ab	4.1 ± 0.6 ab

Mean values \pm SD are shown. Means within a column followed by different letters are significantly different

hypothesis. The N: P ratio (ranging between four and seven under both ambient and elevated CO_2) and P concentration of shoots (between 1.2 and 2.1 mg g^{-1} under both ambient and elevated CO_2), above the deficiency level of 1.0 mg g^{-1} (Reuter et al. 1997) indicated adequate P supply.

Alberton et al. (2005) pointed out that a large source of variation between experiments was due to differences between fungal species and stated that argued choices for fungal species selection are very important. ECM fungi show large intrinsic differences in their N sink strength. Relations between nitrogen availability and fungal carbon use, where increasing N levels result in a shift from fungal growth toward N assimilation, have been noted by Arnebrant (1994) and Wallander (1995). ECM fungi can be classified along a gradient from nitrophobic to nitrotolerant or nitrophilic. Gorissen and Kuyper (2000) showed that the nitrophobic *S. bovinus* could respond to elevated CO_2 , whereas the nitrotolerant *L. bicolor* did not. In their study, there were no significant differences in hyphal length between both species (although *S. bovinus* tended to form more mycelium), but pine seedlings colonized by *S. bovinus* had significantly higher N concentrations in shoots and roots, being above deficiency levels in the

former case, and below deficiency levels in the latter. One important point if one wants to compare the results of this study with that by Gorissen and Kuyper (2000) is the duration of the experiment; in the present experiment it lasted 2 months longer, thereby increasing N-limitation due fungal and plant growth. Calculations, based on the data in Gorissen and Kuyper (2000) showed that *S. bovinus* transferred significantly more N per g ECM than *L. bicolor*; N-uptake efficiency for the former species was 20 and was reduced to 11 under elevated CO_2 , for the latter species 12 and slightly reduced to 11 under elevated CO_2 . Comparison with the present study (N-uptake efficiency of 12–16 under ambient CO_2 , and 8–11 under elevated CO_2) suggests additionally that the previous experiment was done under less N-constrained conditions. The decrease in nitrogen uptake efficiency under elevated CO_2 shows that additional carbon hardly increase N uptake, consistent with the PNL hypothesis.

In the present study, differences between fungal species could very well be explained through one factor only, size of the extraradical mycelium. Differences in C to N exchange ratios in the fungus were clearly of limited importance. When plant growth is limited by nutrients, plants undergo

morphological, and physiological modifications. By changing the functional equilibrium between shoots and roots, plants allocate more C to below-ground structures for nutrient acquisition. If plants allocate more biomass to the organs that are involved in the acquisition of the resource that is currently most limiting, one would predict that elevated CO₂ would decrease shoot: root ratio. Our data are consistent with that prediction. Shoot: root ratios declined under elevated CO₂ from 0.67 to 0.45. A meta-analysis, conducted by Poorter and Nagel (2000) showed that under conditions of severe nutrient limitation, elevated CO₂ led to a decrease in shoot: root ratio. Our experiments showed the largest reduction in shoot: root ratio in plants that were colonized by those ECM fungal species that had the largest extraradical mycelium, lowest N concentration in needles under elevated CO₂ and lowest ¹⁴C incorporation in needles, viz. *L. bicolor* and *H. cylindrosporum*. The ultimate effect of this changed allocation of carbon (and unavoidably of nitrogen!) would be that photosynthesis rates go down, in the end to levels where no effect of elevated CO₂ on shoot biomass or shoot ¹⁴C incorporation was noted (Table 1). The observation that fungal sink strength for N constrains plant response to elevated CO₂ supports our claim that the size of the extraradical mycelium generates potential for negative feedback through PNL (Hu et al. 2006).

The fact that mycelial length, and hence, mycelial biomass did not increase significantly under elevated CO₂ (Table 1) could suggest that plants do have some control over allocation of C to their fungal symbionts. However, increases in the ECM root biomass likely entails increases in fungal biomass on the roots as well. From data by Hobbie and Colpaert (2003) we calculated that fungi contributed 20% to the ECM root mass of pine seedlings (18% under high N, 21% under low N—average for *S. luteus* and *T. terrestris*). Assuming that fungi also contribute 20% to the mass of the ECM in our study, and converting hyphal length into fungal biomass (treating mycelium as an elongated cylinder with a diameter of 2.7 µm and a hyphal density of 1.3 g cm⁻³ (Bloem and Vos 2004), we calculate that the increased allocation below-ground under elevated CO₂ could increase ECM fungal biomass with on average of 32% for most species (64% for *S. bovinus*), remarkably close to the 34% of generalized fungal response in Alberton et al. (2005).

Shoot dry weight increased with only 6% under elevated CO₂, and this was slightly less than the increase in hyphal length (12%). However, both changes were not significant. The increase in shoot weight was also less than reported in Alberton et al. (2005). The low above-ground plant response is mainly due to the low N availability. The experimental system (Petri dish) may not easily allow extrapolation of results into the field. However, this system, which achieves good control over humidity, light availability, temperature, and CO₂ level, has been successfully applied in several previous investigations (Ineichen and Wiemken 1992; Ineichen et al. 1995; Fransson et al. 2007).

In conclusion, fungal sink strength for N, together with low N availability in a restricted growth medium constrained plant growth especially with fungal species with the largest mycelial size. In an experiment where nutrients were not limiting, elevated CO₂ resulted both in a rapid positive response by ECM fungi and in an increase in photosynthetic performance by pine seedlings (Fransson et al. 2007). In nutrient-constrained systems, elevated CO₂ will increase allocation belowground (to mycorrhizal fungi and roots) and enhance nutrient immobilization by mycorrhizal fungi. However, increased immobilization lowers plant N concentration and hence restricts the plant's ability to respond to this elevated CO₂. Mycelial sink strength then generates negative feedback to plant growth under elevated CO₂, as predicted by the PNL hypothesis. If ecosystems are generally 'saturated' (O'Neill 1994) with respect to ECM fungi, calculating the optimal mycelium size, using modeling approaches, taking into consideration soil properties and hyphal distribution in soil is an important challenge.

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